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Transplantation of Fu/HC-Incompatible Zooids in *Botryllus schlosseri* Results in Chimerism

BARUCH RINKEVICH[1], IRVING L. WEISSMAN[2], AND ANTHONY W. DE TOMASO[2,*]

[1] *National Institute of Oceanography, Tel Shikmona, P.O. Box 8030, Haifa 31080, Israel;*
[2] *Department of Pathology, Stanford University School of Medicine, Stanford, California 94305; and Hopkins Marine Station, Pacific Grove, California 93950*

Abstract. The colonial urochordate *Botryllus schlosseri* undergoes a genetically defined, natural transplantation reaction that is controlled by a single Mendelian locus (called the Fu/HC). This Fu/HC-based allorecognition system is initiated when peripheral elements of the vasculature interact on the edges of two asexually expanding colonies. To better understand the spatial organization of the cellular elements responsible for Fu/HC-based allorecognition, we bypassed the normal site of interaction (the ampullae) and experimentally transplanted zooids between Fu/HC-noncompatible *Botryllus schlosseri* pairs. The results show that (1) instead of the expected rejections (tissue necroses) that develop after natural contacts between peripheral blood vessels, the transplanted organs are morphologically eliminated within a few days in conjunction with the normal blastogenic cycle; and (2) donor-recipient chimerism is established after complete morphological elimination of transplanted tissues. These results suggest that Fu/HC-based allorecognition responses in *Botryllus schlosseri* occur exclusively at the ampullae and that once cells have crossed this barrier, they are able to survive and proliferate in the new host colony.

Introduction

Colonies of the urochordate *Botryllus schlosseri* undergo a genetically defined, natural transplantation reaction following allogeneic contacts between peripheral blood vessels. Upon contact, the allogeneic vessels either fuse to form a single colony with a common blood supply or reject each other in a blood-based, inflammatory reaction, after which the two colonies no longer interact. Histocompatibility discrimination resides in a single, highly polymorphic fusion/histocompatibility locus (called the Fu/HC; Scofield *et al.*, 1984) with a large number of codominantly expressed alleles (Rinkevich *et al.*, 1995). Allogeneic fusion occurs between colonies that share at least one Fu/HC allele; in contrast, partners will reject each other if they share no Fu/HC alleles (Bancroft, 1903; Oka and Watanabe, 1957, 1960; Sabbadin, 1962; Scofield *et al.*, 1982).

Several interesting phenomena may occur after two colonies have undergone a Fu/HC-mediated fusion event. In the laboratory, we have observed that, after fusion, the genetic colonial descendants (zooids) from one partner in the chimera cease normal development, and these dying zooids are resorbed by massive phagocytosis, leaving the zooids of the other colony intact (Rinkevich and Weissman, 1987). This phenomenon, called colony resorption, occurs at the end of the synchronized weekly blastogenic cycle in which the old generation of zooids dies through a programmed apoptotic event (Lauzon *et al.*, 1993). We have used genetically defined laboratory colonies to show that the ability of one colony to resorb another is consistent, and that different genotypes can be grouped into a reliable hierarchy. However, analysis of the segregation of these traits in defined crosses suggests that it is a complex phenomenon involving at least several loci, possibly including the Fu/HC locus (Rinkevich *et al.*, 1993). Thus, resorption in *Botryllus* has been compared to the minor histocompatibility loci seen in the vertebrates (Rinkevich, 1993).

Several laboratories have also shown that, after fusion, each colony can exchange germ and somatic stem cells which are then able to survive and replicate in the allogeneic colony. In fact, as first described by Sabbadin and Zaniolo (1979), the germ cells from one colony can actually completely parasitize the other colony in a fused

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* To whom correspondence should be addressed. E-mail: tdet@leland.stanford.edu

chimera, such that only one of the genotypes is represented in the mature germ cells. This can occur up to a month after two fused colonies have been experimentally separated, demonstrating that cells from one colony survive and proliferate in the other (Sabbadin and Zaniolo, 1979; Pancer *et al.*, 1995; Stoner and Weissman, 1996).

These two postfusion events appear to be opposite in nature and are difficult to reconcile. On one hand, the resorption phenomenon suggests that in an allogeneic chimera there is still a form of directed allorecognition occurring, and the ability to demonstrate a hierarchy among laboratory-bred colonies suggests a genetic component to this process. Conversely, the precursors of germ and somatic cells clearly can proliferate inside an Fu/HC-matched, allogeneic colony, suggesting that there is not a secondary form of allorecognition occurring, particularly on a global scale. This is further complicated by recent observations that, in a fused colony in which one of the partners had been resorbed, only the genotype of the resorbed partner was present in the germ line. Thus a somatic loser (by resorption) can be a gametic winner (by successful germ cell competition) (Pancer *et al.*, 1995; Stoner and Weissman, 1996).

The above observations led us to ask whether any allogeneic effector systems are present throughout the entire colony. To investigate this question, we decided to circumvent the natural manifestation of incompatibility, which occurs when peripheral blood vessels interact, by grafting whole zooids between colonies with rejecting Fu/HC genotypes. Results from these experiments provide further evidence that Fu/HC-mediated allorecognition, the rejection effector system, or both, may be restricted to the ampullar tips, suggesting that allorecognition responses are not a colony-wide phenomenon in *Botryllus*.

Materials and Methods

Animals

We used colonies of *B. schlosseri* that were originally collected from the Monterey Marina (California) but have been growing in the laboratory at Haifa, Israel, and at Hopkins Marine Station, Pacific Grove, California. Colonies were born and reared separately on glass slides (5 × 7 cm) in glass staining racks within 17-l tanks as described (Boyd *et al.*, 1986). Subclones of individual colonies were Fu/HC phenotyped using a cut colony assay (Rinkevich *et al.*, 1993), and rejecting pairs of colonies were identified. These subclones were not used in the transplantation assays.

Transplantation

Reciprocal transplantation of whole zooids between rejecting *B. schlosseri* pair genets was performed during developmental stages A–C of the blastogenic cycle (described in Milkman, 1967). A longitudinal incision was made (with a thin needle) between the atrial and branchial siphons of a single zooid (highlighted in Fig. 1b). Both edges of the incision were then retracted with fine forceps. The incision went through the tunic and upper body wall of the individual zooid, and retraction of the edges exposed the body cavity, which contained the body of the zooid, the bud, and the gonads (see Berrill, 1941, for an in-depth description of *B. schlosseri* anatomy). The zooid was lifted and removed with a fine needle and forceps, leaving behind a "cup" consisting of the outer and lower tunics lined with epithelium, connective tissues, cut blood vessels, and, in many cases, all or part of the buds and the gonads. Bleeding from the cut blood vessels (either from the removed zooids or from the leftover vessels) was always seen. The removed zooid was then replaced by another zooid taken from a naive subclone of a rejecting genet, or in the case of controls, from another subclone of the same genet. A control zooid and the experimental zooid were transplanted within the same system, separated by 1–3 zooids (Exps. I–III, Table IA) or in adjacent systems (Exps. IV–VI, Table 1B). The transplanted zooids were carefully inserted within the empty cups, one zooid per cup, pushed slightly inside with the blunt end of a forceps, and covered with the almost enclosing, cut surface of the outer tunic layer. In all cases of transplantation a mixture of blood cells coming from the introduced zooid and from the local bleeding was documented. Bleeding stopped within a few minutes.

Tissue sampling

Amplified fragment length polymorphism (AFLP) analysis was performed on tissue samples from Exps. I–III. Exps. IId and IIId died prematurely. Sampling was usually done 3–4 weeks after the final transplantation. Before tissue was sampled, each subclone was carefully checked under a dissecting microscope to determine whether the transplanted zooids had been completely resorbed. In one case (Exp. IIc, Table I) the whole subclone, which started to degenerate, was isolated as is. All other seven subclones were photographed, their general structure was outlined, and three to seven tissue samples per subclone were separated with a razor blade and individually snap-frozen in liquid nitrogen.

Amplified fragment length polymorphism (AFLP) analysis

All enzymes were purchased from New England Biolabs (Beverly, Massachusetts), and the chemical reagents were purchased from Sigma (St. Louis, Missouri). Oligonucleotides were synthesized at the PAN facility at the Stanford University Medical School. Frozen tissue samples were ground to a fine powder with a mortar and pestle. DNA was extracted on silica columns (Nucleo-

Table I

Experimental procedures and major results for allogeneic and isogeneic zooid transplantation

Experiment #	Allogeneic* combination	Pair #	Donor genotype	Initial size (# zooids)†		Major outcomes‡
				Recipient	Exp. system	
A = One-month experiments						
I	1 vs. 2	a	1	23	10	4 repeated zooid transplantations (within 21 days from first event), all resorbed together with isogeneic controls and before the takeover phase of blastogenesis. No single case of POR. The ramet died during the 5th transplantation, before completion.
		b	1	20	9	Same as Ia.
		c	2	20	7	6 sets done within 1 month. Resorption of transplanted allogeneic and isogeneic zooids in all cases started after transplantation and terminated during takeover. No POR. Sampled for AFLP analysis.
		d	2	11	7	Same as Ic; sampled for AFLP analysis.
II	3 vs. 4	a	3	30	13	Same as Ic; sampled for AFLP analysis.
		b	3	32	10	Same as Ic; sampled for AFLP analysis.
		c	4	16	7	The first 4 sets of transplantations as in Exp. Ia. During the 5th set (day 25), the ramet degenerated and was sacrificed for AFLP analysis as one sample. No POR.
		d	4	15	10	Morphology same as Ic, died on day 25, no further sampling.
III	5 vs. 6	a	5	31	7	Same as Ic; sampled for AFLP analysis.
		b	5	13	6	Same as Ic; sampled for AFLP analysis.
		c	6	21	10	Same as Ic; sampled for AFLP analysis.
		d	6	26	12	Same as Ic; died prior to sampling.
B = Two-month experiments						
IV	7 vs. 8	a	7	27	11	Same as Ic; but zooids transplanted within two months. No AFLP sampling was done on these experiments.
		b	8	19	9	Same as IVa.
V	9 vs. 10	a	9	22	8	Same as IVa.
		b	10	18	7	Same as IVa.
VI	11 vs. 12	a	11	30	9	Same as IVa.
		b	12	31	8	Same as IVa.

* Each of the 12 genets was used in only one set of experiments.

† The size of the recipient subclone at the day of the first transplantation, and the size of the experimental system within the recipient subclone on which both transplanted zooids (experimental and control) were introduced. Follow-up transplantations were performed on daughter zooids, produced through blastogenesis from intact buds, of these same systems.

‡ POR, point of rejection; AFLP, amplified fragment length polymorphism.

bond C+T Kit, Macherey Nagel, Duren, Germany) using proprietary buffers according to the manufacturer's instructions. AFLPs were performed as described previously (Vos *et al.*, 1995). Briefly, 200 ng of DNA was cut to completion with restriction enzymes *Eco* RI and *Mse* I for 2 h at 37°C in a 30-μl reaction volume. Oligonucleotide adaptors, 1 mM ATP, and T4 DNA ligase were then added (total volume, 40 μl), and the incubation was continued for 3 h. The DNA was preamplified with one selective nucleotide on each primer (Eco RI = A; Mse I = T). The preamplification mix was diluted 1:20 and 3 μl was used for AFLP fingerprinting with each primer containing three selective nucleotides (Eco RI = ATg; MseI = TCg). The Eco RI primer was end-labeled with ^{33}P-ATP (New England Nuclear) using polynucleotide kinase. PCR reactions were diluted 1:1 in stop solution (98% formamide, 10 m*M* EDTA pH 8, 0.1% bromophenol blue, 0.1% xylene cyanol), denatured for 5 min at

95°C, and resolved on a standard sequencing gel at 70 W for 2.5 h. Gels were dried, and the autoradiograms were exposed for 36 h. Each experiment was repeated two times to ensure that the AFLP fingerprints were consistent. In some sets, different primer sets were used, and the results were equivalent (not shown).

Results

Twelve *B. schlosseri* colonies were organized into six rejecting pairs, providing six independent experiments (see Table 1). Within each pair, two types of transplantations were done in parallel (described in the Methods). Zooids were reciprocally transplanted between the two rejecting colonies to make allografts; and zooids were transplanted from another ramet of each colony to make isografts. Allograft and isograft transplantations were carried out within a single system of the recipient, usually a few zooids apart (see Fig. 1). There were no observed differences in response if allografts or isografts were done independently (not shown).

The initial transplantation procedure was followed by four to six sequential transplantations over the course of 1 (Exps. I–III) or 2 (Exps. IV–VI) months (Table 1). Multiple transplantations into the same ramet were done to test for the induction of a rejection response after repeated exposures to the same allogeneic tissue. Transplantations were also done at different points of the blastogenic cycle (Milkman, 1967) to test for any variability in the alloresponse. In Exps. I–III (Table 1A) the subsequent transplantation was performed immediately after the takeover phase of blastogenesis (Milkman, 1967), while in Exps. IV–VI (Table 1B), one full blastogenic cycle separated the two sequential transplantations.

Apart from the zooids that were completely excised during the transplantation procedure, other zooids in the experimental system and in all the other systems within the same colony were usually not affected. A few hours after transplantation, the implanted tissues were covered by the matrix of the cut tunic, sealing them within the recipient colony.

Under normal conditions in a *Botryllus* colony all zooids and buds are connected by vascular outgrowths to the colonial circulatory system (Milkman, 1967). Vascular anastomoses were not observed in any of the 114 allogeneic and isogeneic zooids transplanted in these experiments. Although there was no long-term vascularization, hemocytes from the donor and recipient were in contact for several hours following the transplantation procedure: there was bleeding from the cut vasculature of the recipient colony, as well as from the open circulatory system of the donor zooid. This allowed mixing of host hemocytes with donor hemocytes and tissue for several hours. After 12–24 h, the disconnected blood vessels of the colony regenerated, sometimes forming a circular pattern of blood vessels around the transplanted zooid (not shown). Thus the allogeneic interactions in these experiments can be summarized as follows: the donor zooid was in contact with the recipient hemocytes, while the donor hemocytes were allowed to mix with the colonial circulation of the recipient, analogous to injection of hemocytes across a Fu/HC incompatible barrier.

Within 24 h, many of the implants were completely covered by the upper tunic wall (Fig. 1a). In some cases, especially when a large zooid was transplanted, part of the zooid extended out of the "cup," closed around by a "collar" made of the colony epidermal wall (not shown). Within the next few days, all of the implants degenerated, a phenomenon coinciding with the regular colony blastogenesis (Fig. 1b) in which all zooids go through a systemic programmed apoptotic cycle, followed by massive phagocytosis (Lauzon *et al.*, 1992). There was no observable difference between resorption of isografts or allografts (Fig. 1a, c). For instance, in Figure 1c, the isograft appears to be resorbing faster than the allograft. Any variations in the time scale of resorption (2–5 days) were related to the blastogenic cycle. More importantly, no visible points of rejection (POR; Table 1) were ever observed in these transplantations (Fig. 1a, b), although natural contact assays done on other ramets of the same six pairs of genets (Table 1) always resulted in typical, distinct POR within 24–48 h after first ampullae contacts (not shown). After the takeover phase of blastogenesis, all or most parts of the allogeneic and isogeneic tissues were resorbed and had disappeared, leaving behind a clear tunic matrix or a space occupied by the new generation of developing zooids (Fig. 1b). In some cases, remnants were found trapped in the bare tunic (Fig. 1d) in a manner similar to that recorded in regular colonies (Rinkevich and Weissman, 1987).

Two major morphological variations in the outcomes of transplantation were documented, although neither was related to the type of transplant (autograft or allograft). The first was a partial resorption of an intact zooid bordering the area of transplantation (Fig. 1c), probably resulting from the experimental manipulation of zooid excision and transplantation. Morphological resorption as a result of stress conditions has already been documented in *Botryllus* (Rinkevich *et al.*, 1993). This type of partial resorption (Fig. 1c) occurred within 24 h of zooid transplantation, but was never completed before the takeover phase (Lauzon *et al.*, 1992) of blastogenesis. The second type of variation was the resorption rate of the implant. In some cases, the implants were resorbed completely or mainly within 24 h of transplantation (Fig. 1e, f). Where partial resorption was recorded (Fig. 1f), the leftover parts remained within the tunic for the whole blastogenic cycle and were completely resorbed only during the takeover stage.

To analyze the possibility for donor cell proliferation

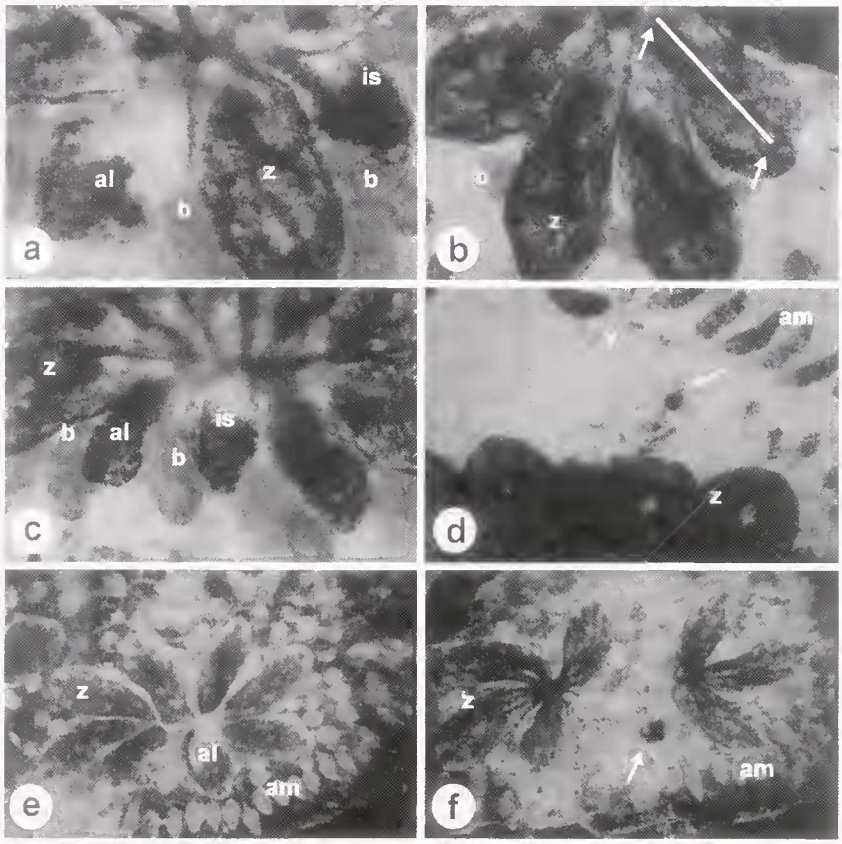

Figure 1. Morphological outcomes for zooid transplantation in Fu/HC-noncompatible colonies of *Botryllus schlosseri*. al = allogeneic transplant, am = ampulla, b = bud, is = isograft, v = blood vessel, z = zooid. Length of each zooid = 2 mm. (a) Results from Exp. 1c (Table 1): The second set of allogeneic and isogeneic grafts, 24 h after implantation, when the colony is at blastogenic stage C. (b) The same area, photographed 72 h later, following a takeover event. Both implanted zooids are completely resorbed, and there is no difference between control and allograft; the zooid between them as well as adjacent buds are unaffected. After the takeover process, all transplanted materials were morphologically eliminated, no point of rejection (POR) is observed. Note the top right zooid: the incision for transplanting the zooids is highlighted; white arrows point to the atrial and branchial siphons, and the white line highlights the incision made for the transplantation procedure (see Methods). (c) Results from Exp. 1d (Table 1) 24 h after first set of implants was established. The control isograft appears to be resorbing faster than the allograft. The zooid to the right of the isograft is also partly resorbed. The colony is at blastogenic stage C, and the developed buds in the area of implantation are not affected. (d) Exp. 1b, after resorption of most of the allogeneic implant. Resorption is not complete, and a remnant of the graft (arrow) is trapped in the tunic matrix. (e, f) Results from Exp. 5a (Table 1): The third set of allografts, immediately after implantation (a), and 24 h later (f). By 24 h, most of the graft has been resorbed and only a small part of it (arrow) is left. This remnant remained for an additional 3 days, and was resorbed during the takeover stage of blastogenesis.

in the recipient (Sabbadin and Zaniolo, 1979; Pancer *et al.*, 1995; Stoner and Weissman, 1996), all the available subclones from the 1-month experiment (five or six consecutive transplantation events; Table 1A) were sampled (1–7 fragments/subclone) 3 to 4 weeks after complete resorption of the donor's zooids (except for Exp. 1c, see Table 1). The genotype of the recipient was then analyzed using amplified restriction fragment polymorphisms (AFLPs; Vos *et al.*, 1995). AFLPs identify DNA polymorphisms between individuals; these polymorphisms can then be used as molecular genetic markers. AFLP polymorphisms are often single base-pair substitutions, and since these are the most abundant polymorphisms available, a large number of AFLP genetic markers can be identified, even between closely related colonies (Fig. 2). In these experiments, 6 to 12 unique AFLP markers were first identified in the naive donor subclones. Following a transplantation, we then looked for the unique donor AFLPs in samples of recipient DNA.

Tissue samples were taken such that the transplanted area (including the relevant system of zooids and the surrounding tissue matrix) was isolated from the rest of

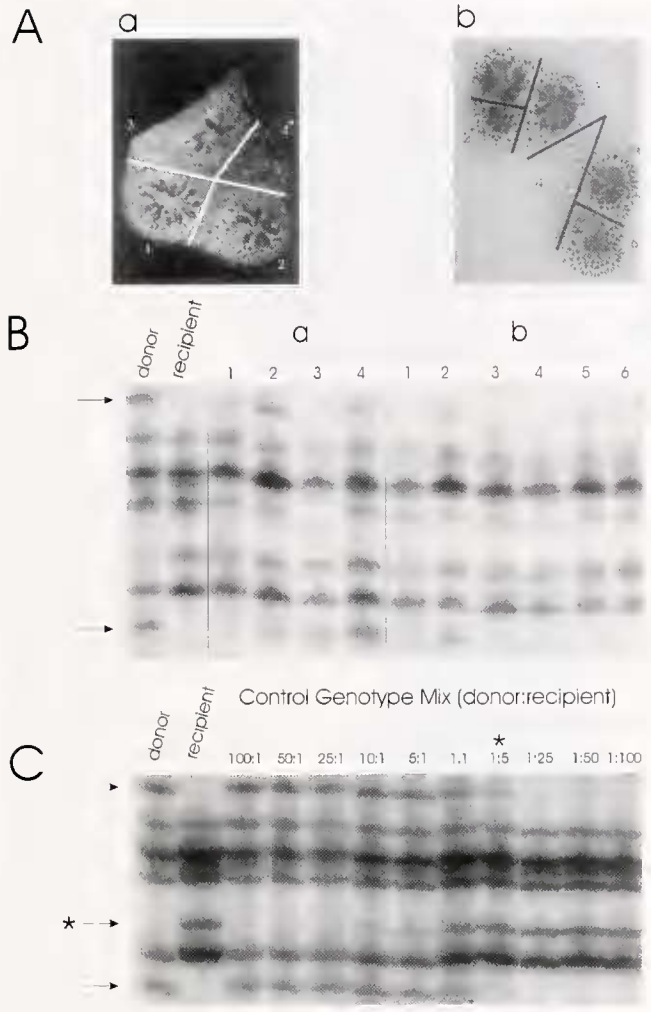

Figure 2. Amplified fragment length polymorphism (AFLP) fingerprints reveal the presence of donor alleles in the recipient after zooid transplantation. Panels a and b correspond to Experiment II, pair number a and b, in Table 1. (A) Photographs of the recipient ramets after six sets of zooid transplantation and before subcloning. The areas sampled for DNA extraction and AFLP analysis are delineated and numbered. An asterisk indicates the site to which the donor zooids were originally transplanted; boxed numbers indicate a sample that contained peripheral vascular tissue only. (B) AFLP fingerprints of the donor, the naive recipient, and samples taken from the recipient after transplantation, as shown in A. Arrows indicate polymorphic AFLP alleles present in the donor and absent in the naive recipient. The appearance of these bands in the recipient ramets after transplantation indicates the presence of donor DNA. The recipient bands are invariable, indicating that the recipient genotype is stable and the resulting animal is a chimera. A section of the complete gel is shown for clarity. (C) A control experiment showing the independent sensitivity of each polymorphic AFLP donor locus in known mixtures of genotypes. DNA from the donor and naive recipient were mixed in the indicated ratios and the corresponding AFLP fingerprints were produced. The closed arrows indicate the same polymorphic donor loci as shown in B. The asterisk denotes a DNA mixture in which the top allele is present and the bottom allele is very faint. In this section of the gel a recipient locus can also be seen (arrow plus asterisk) and demonstrates that polymorphic loci can differ as much as 10-fold in their ability to be amplified from low concentrations.

the subclone. Then each subclone was further divided into several parts, including bare-tunic ampullae zones. Figures 2 and 3 depict the sampling details for seven subclones. One subclone (Exp. IIc) was not separated, but was used whole for a single AFLP analysis. In seven of the eight subclones (Fig. 2, Fig. 3 except Exp. IIIc) donor AFLPs were clearly documented in all samples, including zooid-free areas and zones away from the transplantation areas. However, the degree of chimerism was not consistent among all parts of the recipients; different regions showed a higher or lower percentage of the donor genotype. In other words, not all of the donor AFLP markers were amplified from each recipient DNA sample (Fig. 2b). This is because the ability to amplify a particular AFLP marker from a mixed sample of DNA is unique for that marker; some AFLP markers can be identified when they represent less than 1% of the total DNA, while others are less sensitive (Fig. 2c). Thus, if the amount of chimerism is very low (*e.g.*, donor DNA $< 1\%$ of the total DNA in the sample), only the most sensitive donor AFLP marker will be identified (see Fig. 2c). In contrast, in a sample that contains a higher amount of donor cells, the other, less sensitive AFLP markers will also be identified (Figs. 2 and 3). This provides a nice tool for estimating the amount of chimerism, but because the AFLP technique includes an initial PCR amplification of the total genomic DNA sample (Vos *et al.*, 1995), these comparisons are relative and can only be made in a side-by-side comparison to the controls (Fig. 2b, c).

Chimerism was detected globally in the recipient colonies more than 3 to 4 weeks after the final transplantation, and the degree of chimerism was variable in different samples. This suggests that donor cells proliferated in the host colonies; the small number of hemocytes from several transplanted zooids are unlikely to have been passively circulated and then detected several weeks later, after several rounds of blastogenesis.

Exp. IIIc (Table 1; Fig. 3) was the only case in which we did not observe the donor genotype in any sample, although in the reciprocal tests (Exp. IIIa, b; Fig. 3) the other partner's DNA was clearly evident in all tissue samples. This is reminiscent of the directionality observed in resorption, where hierarchies were demonstrated in laboratory-reared colonies (Rinkevich *et al.*, 1993).

In summary, chimerism between the donor and recipient appeared to be the rule rather than the exception in these experiments. In 11 of 12 cases, donor AFLP markers could be identified in the recipient more than 3 to 4 weeks after the donor transplant had been eliminated. Furthermore, high amounts of chimerism were detected throughout the recipient colony. This suggests that once donor cells have crossed the ampullar barriers, they are able to survive and proliferate in the recipient colony, and are not being eliminated by an Fu/HC-based, or any other, allorecognition system.

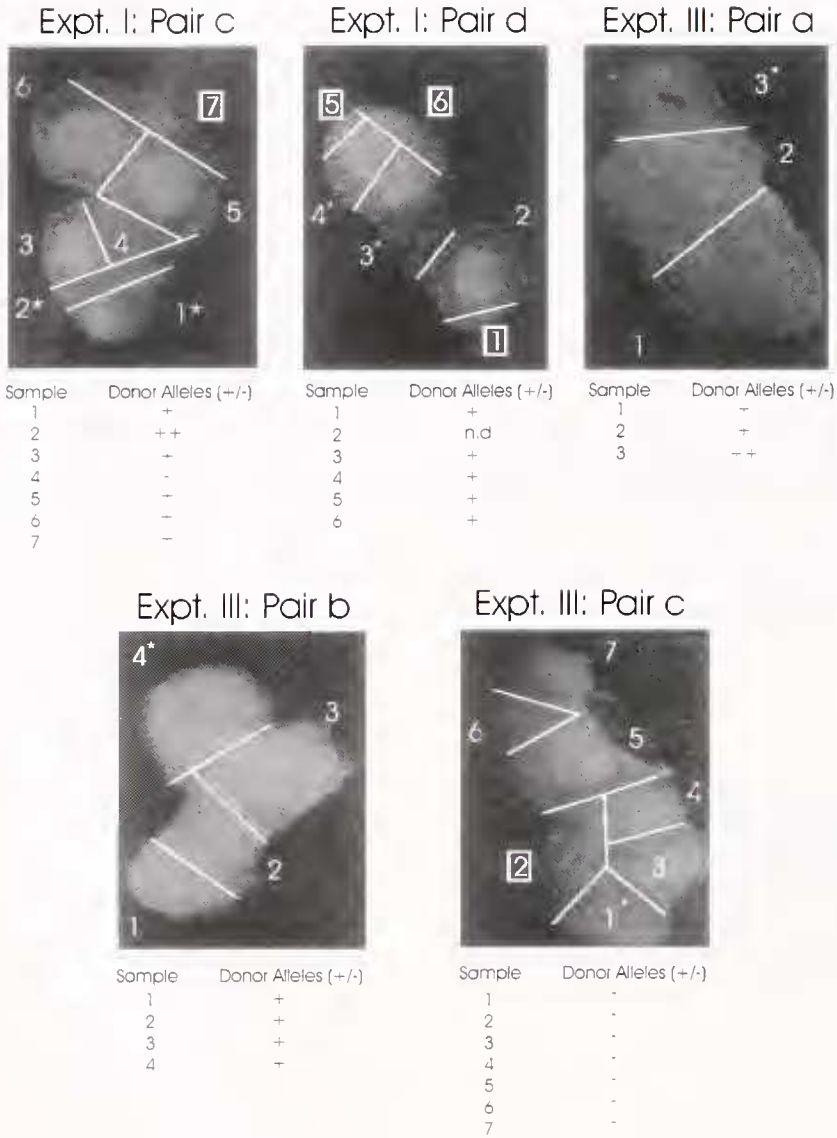

Figure 3. A summary of amplified fragment length polymorphism (AFLP) data from experiments described in Table I. Photographs show five recipient ramets after transplantation. The areas sampled for DNA extraction and AFLP analysis are delineated and numbered. Asterisks indicate the sites to which the donor zooids were originally transplanted; boxed numbers indicate a sample that contained vascular tissue only. Because of the independent sensitivity of each polymorphic donor locus (illustrated in Fig. 2C), not all of the polymorphic donor loci were seen in each of the transplanted recipient samples. Samples were scored as (++) if they contained over 50% of the polymorphic donor loci, (+) if under 50%, and (−) if none were seen. "n.d." indicates that the sample was not determined.

Discussion

This study produces two interesting results. First, zooid transplantation between noncompatible Fu/HC *B. schlosseri* genotypes does not result in the typical formation of visible PORs (reviewed in Weissman *et al.*, 1990). Rather, transplantation is followed by a morphological resorption similar to the allogeneic resorption that takes place after fusion between Fu/HC-compatible colonies (Rinkevich and Weissman, 1987). Second, the Fu/HC-noncompatible genotypes continue to thrive within the host, even away from the transplantation zone; this phenomenon had previously been recorded only from Fu/HC-compatible encounters (Sabbadin and Zaniolo, 1979; Pancer *et al.*, 1995; Stoner and Weissman, 1996). Circumvention of the natural contact areas in *B. schlosseri* demonstrates that once cells have crossed the allogeneic barriers at the ampullae, they are no longer subject to elimination from an

Fu/HC-based allorecognition system, as demonstrated by the ability of cells to survive and proliferate in a completely allogeneic host.

The idea that Fu/HC-based allorecognition responses in botryllid ascidians could be limited to the ampullae is not new. The differences in rejection mechanisms between *B. scalaris*, *B. primigenus*, *B. schlosseri*, and several species of the genus *Botrylloides* have been postulated to reflect the change in the cells responsible for allorecognition from freely circulating (in *B. scalaris*), to residing outside the tip of the ampullae or in the tunic (*Botrylloides*) (reviewed in Saito *et al.*, 1994). Further evidence for this theory comes from experiments on *Botrylloides fuscus*, where fusion always occurs in a cut surface assay (where nonampullar vasculature elements are brought into contact), even between colonies that reject each other at the growing surface (*i.e.*, ampullar contacts; Hirose *et al.*, 1994). Furthermore, recent fine-scale EM observations on rejection in *Botrylloides simodensis* and *B. fuscus* strongly suggest that allorecognition responses are limited to the tunic in these species (Hirose *et al.*, 1997).

However, the situation in *B. schlosseri* is not as clear. There are no detailed reports of cut surface assays from this species. In our hands, cut surface assays are not definitive; we often see structures that look like POR between rejecting colonies, but this has never been consistent, nor as vigorous as the response at the ampullae (Rinkevich, 1992; unpubl. data). Furthermore, in experiments described by Sabbadin (1982), secondary buds were transplanted to zooid-free colonies that were both Fu/HC isogeneic and allogeneic, and the ability of these transplanted buds to vascularize, develop, and mature was analyzed. Although the survival of the transplanted bud in an isogeneic colony was clearly different from that in an allogeneic colony, there was no mention of the typical rejection response observed at the ampullae. Since, in Monterey *B. schlosseri*, rejection occurs prior to complete tunic fusion (see Saito *et al.*, 1994), allorecognition elements may be restricted to the vascular epithelia, or to a small subset of cells that did not contact the transplanted zooids in these experiments. It is also possible that Fu/HC-based allorecognition events were occurring but were not detected. Typical, visible POR formation may be a complex event, involving cells and signaling pathways not available at the interface between zooid and vasculature. There is a difference between recognition and response, and since none of the molecules involved in allorecognition in the botryllid ascidians have been identified, only the response is truly assayed. Thus, in these experiments, recognition could be occurring, but without a subsequent response, which would be analogous to effector cell function in the adaptive immune system of the higher vertebrates. In that system, two signals are often required for a response; recognition by the effector cell, and a costimulatory signal from another cell. If the costimulation signal is absent, there is no effector response (reviewed in Matzinger, 1994).

The ability of donor cells to survive and be detected in the recipient for 3 to 4 weeks after transplantation is the best evidence that Fu/HC-based allorecognition responses are spatially segregated in *B. schlosseri*. But the specific components of the Fu/HC allorecognition response that are spatially segregated are unknown; they could be cells involved in the recognition of allogeneic colonies, in the downstream effector mechanisms responsible for POR formation, or both.

This leads us to ask why the site and severity of fusion/rejection reactions have changed between the different botryllid ascidians (discussed above). One answer might be to lower the costs (*e.g.*, cell death, loss of ampullae) of allogeneic reactions. Another possibility is to limit the amount of cell-cell interaction during an allogeneic reaction, thereby limiting the ability of any cells to cross to an allogeneic colony and begin to parasitize it. Questions like this will be answered only when the Fu/HC and receptors have been characterized at a molecular level, which will allow the cells involved in allorecognition to be identified.

These results also bring up intriguing questions about the resorption phenomenon hypothesized to be a global allorecognition system analogous to minor histocompatibility in the vertebrates (Rinkevich, 1993). In previous studies, we have shown that some cells, notably those that are able to parasitize the germ line of the host colony, are not resorbed (Pancer *et al.*, 1995; Stoner and Weissman, 1996). But in these studies, the cells that survived the resorption event had at least one Fu/HC allele in common with the host colony and were therefore acceptable according to the rules of Fu/HC-based colony specificity. In this study, Fu/HC-mismatched cells continued to survive in a host colony, although we do not know what type of cells they were, or whether they would have been able to parasitize the germline of the host colony. Resorption may not include cells circulating in the vascular system, but the ability of cells to survive and proliferate in an allogeneic colony (Fu/HC-matched, or not) makes us wonder what the effector mechanisms responsible for resorption are responding to. Why would it be that the zooids are resorbed, but cells circulating in the bloodstream—especially those that could parasitize the host colony—are not? Since we did not assay for chimerism after a single transplantation event, it might be postulated that the repetitive transplantations in these experiments are either overwhelming, or inducing tolerance in, the resorption machinery. This seems unlikely since, in previous experiments, one partner of a natural (*i.e.*, Fu/HC-matched) chimera could resorb another, even when the losing (resorbed) partner was three times larger. Further-

more, resorption could occur months after a chimera had become established (Rinkevich and Weissman, 1987).

The ability of transplanted cells to survive and proliferate in an Fu/HC-incompatible colony, while revealing something of alloresponses in *B. schlosseri*, may also seem to be a complete artifact of a laboratory experiment. The existence of the Fu/HC-based allorecognition system and the maintenance of such incredibly high levels of polymorphism at the Fu/HC locus have been hypothesized to limit fusion, and the possibility of subsequent germ and somatic cell parasitism, to kin (Buss, 1982; Pancer *et al.*, 1995; Stoner and Weissman, 1996). Yet, although this system has been investigated for many years, the ability of the Fu/HC-based rejection response to block the transfer of cells capable of germ or somatic cell parasitism during an allogeneic interaction has never been directly tested. Interestingly, cells that appear to be able to transfer from one colony to another during a rejection reaction have recently been observed (Rinkevich *et al.*, 1998), and we have found possible cases of parasitism in unfused, adjacent colonies in the wild (Stoner and Weissman, 1996). While much work remains to be done to confirm these results, the ability of cells to survive and proliferate in an Fu/HC-incompatible colony may not be a completely unnatural phenomenon, particularly if a Fu/HC-based rejection reaction does not provide complete protection against somatic or germ cell parasitism. The ability of cells to survive in Fu/HC-mismatched allogeneic colonies has interesting implications for the study of allorecognition in *B. schlosseri*.

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